*J. Phycol.* **44,** 71–76 (2008)

DOI: 10.1111/j.1529-8817.2007.00453.x

# ISOLATION OF A NON-PHAGE-LIKE LYTIC VIRUS INFECTING *AUREOCOCCUS ANOPHAGEFFERENS*[1]

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**We have been working to characterize viruses that infect the HAB-forming pelagophyte *Aureococcus anophagefferens* Hargraves et Sieburth. Field samples were collected during brown-tide events in 2002 and tested for the presence of lytic agents. Here, we describe a recently isolated, lytic virus-like particle (VLP) that is morphologically similar to particles observed in thin sections of infected *A. anophagefferens* cells from natural samples. TEM and SEM have revealed VLPs consistent with the morphological characteristics of previously described Phycodnaviridae. Large icosahedral particles (~140 nm) of similar shape and morphology dominate cell lysates and are accompanied by smaller phage-like particles and heterotrophic prokaryotes that appear to be incurable from our cultures. To determine which of these particles interacts with the *Aureococcus* cells, we preserved cultures during the early stage of infection so that SEM could be used to visualize those particles that attach to the surface of naïve cultures. SEM revealed that 63% of the large icosahedral-shaped particles attached to *A. anophagefferens* cells after only 30 min of exposure, while no significant frequency of attachment to the alga was observed for the phage-like particles. The results of these observations are in contrast to previous studies, where phage-like particles were reported to infect cells. When considered in conjunction with field observations, the results suggest that this newly isolated virus represents the dominant virus-morphotype associated with bloom collapse and termination.**

***Key index words:* *Aureococcus anophagefferens*; Phycodnaviridae; phytoplankton mortality; SEM**

***Abbreviations:* DOM, dissolved organic matter; FSU, fluorescence spectral units; MOI, multiplicity of infection; VLP, virus-like particle**

Brown-tide events caused by *A. anophagefferens* have been observed in mid-Atlantic estuaries since 1985 and can be devastating to the surrounding environment because they attenuate sunlight that reaches submerged photosynthetic organisms (Bricelj and Lonsdale 1997, Gobler et al. 2005). One common photosynthetic organism affected is eelgrass, an important food source and habitat for members of higher trophic levels (Bricelj and Lonsdale 1997). Previous studies have investigated the factors that potentially influence *A. anophagefferens* bloom dynamics and have suggested that bioavailable dissolved organic matter (DOM) promotes blooms. As such, increased virus densities during a bloom event may promote bloom growth by providing a source of DOM through cell lysis (Gobler et al. 2004). Companion studies have also examined factors that may limit bloom proliferation. In these studies, observations of

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thin-sectioned natural samples of *A. anophagefferens* taken during bloom events have revealed visibly infected cells, with a frequency of nearly 40% toward the end of the bloom event (Gastrich et al. 2004). These findings have led researchers to believe that viruses play an integral role in bloom dynamics, regulation, and potentially termination. Previous studies (Milligan and Cosper 1994, Garry et al. 1998) have reported smaller, bacteriophage-like tailed particles in association with lysis of cultures of *A. anophagefferens*. These findings were not consistent, however, with subsequent published TEM data, which showed that natural samples of *A. anophagefferens* contain particles that are ~140 nm in diameter and icosahedrally shaped (Gastrich et al. 2002, 2004). These observations as well as the recent isolation of bacteria that also lyse *A. anophagefferens* (Frazier et al. 2007) have made the reexamination of this host-virus system a priority. To this end, we set out to identify particles consistent with those observed in nature that may be consistently associated with the lysis of *A. anophagefferens* cells.

## METHODS AND MATERIALS

*Sample collection and culturing.* Water samples from a variety of locations have been collected as part of an ongoing study of the *A. anophagefferens* virus-host dynamics (see Gastrich et al. 2004). For the current study, samples collected at Quantuck Bay (40°49′ N, 72°37′ W) and Great South Bay (40°25′ N, 73°08′ W), New York, USA, were used to screen for naturally occurring viruses that can infect *A. anophagefferens*. Water samples were collected as previously described (Gobler et al. 2004), and the virus-size class (30 kDa, 0.20 μm) concentrated using an Amicon M12 ultrafiltration system (Wilhelm and Poorvin 2001). Virus concentrates were then returned to the lab and screened for lytic activity against cultures of *A. anophagefferens* CCMP 1784. *A. anophagefferens* cells were maintained in an alteration of a previously described brown-tide medium (Cosper et al. 1993) made with water from the Sargasso Sea (reduced to 28 psu with Milli-Q purified water, Academic Gradient A10 Biocel Synthesis; Millipore, Sundbyberg, Sweden) and with a final selenium concentration of 10 μm. Cultures were maintained at 22°C and under a 12:12 light:dark (L:D) cycle (~50 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$) in 50 mL glass culture tubes. Samples were screened daily for plankton growth (via chl production) using a Turner Designs TD-700 fluorometer (Sunnyvale, CA, USA) equipped with an in vivo chl *a* filter set (excitation $\lambda$ = 340–500 nm; emission $\lambda$ = >665 nm).

*Virus infection and lytic cycle length.* During screening, a sample from Quantuck Bay was demonstrated to contain a filterable lytic agent infectious to *A. anophagefferens*, and three samples from Great South Bay contained nonfilterable lytic agents (later identified as bacteria) also infectious to *A. anophagefferens* cells. The infectious agent isolated from the Quantuck Bay sample could pass though a low-protein-binding, 0.2 μm nominal-pore-size polycarbonate filter (GE Osmonics Inc., Labstore, Minnetonka, MN, USA), while those from the Great South Bay samples could not (see Frazier et al. 2007). To determine an approximate length of the lytic cycle, a simple one-step growth curve was performed. Cells were infected with filtered virus at a minimum multiplicity of infection (MOI) of 1 (empirically determined, data not shown), and cell growth (or death), as compared to controls, was monitored over time as fluorescence spectral units (FSUs). To ensure that fluorescence was a valid measure of cell growth (not complicated by the process of infection and cell lysis), an additional assay was conducted at the same time. Twenty-one cultures of *A. anophagefferens* were initiated at the same time and allowed to recover from the stress of transfer. Control cultures received either no lysate ($n$ = 3) or killed lysate (autoclaved to kill any remaining biotic factors, $n$ = 3). Prior experiments indicated no difference between these two types of controls (data not shown). The remaining 18 cultures were inoculated at the same time from the same stock of lytic agent with 100 μL of 0.22 μm filtered lysate (again, minimum MOI of 1). After taking an FSU reading of all the cultures at specific time points (starting with inoculation time), three random treatment cultures were sacrificed, and cell abundance was determined by manual counting via epifluorescence microscopy. These cultures were fixed with glutaraldehyde (2% v/v final), and 2 mL of sample was collected onto 25 mm diameter, 0.22 μm nominal-pore-size black polycarbonate filters (Osmonics Inc.). Autofluorescencing cells were enumerated via epifluorescence microscopy using a Leica DMRXA microscope (Leica Microsystems, Wetzler, Germany). Three control cultures and three random treatment cultures were allowed to run until the end of the experiment.

*Sample preparation and TEM analysis of free VLPs (negative stain).* Virus-mediated cell lysates, generated from unhindered infections, were spotted onto carbon-coated collodion (2%; Electron Microscopy Sciences, Hatfield, PA, USA) films atop 400-mesh electron microscope copper grids and allowed to sit for 5 min (providing for attachment via Brownian motion). Grids were then stained with 0.75% uranyl acetate for 1 min and rinsed three times with glass distilled water. Samples were viewed with a Hitachi H-800 TEM (Hitachi High Technologies America Inc., Schaumburg, IL, USA) with an accelerating voltage of 100 KeV. This methodology provided a means to determine the presence and examine the basic morphology of the suspected infectious agent.

*TEM—sample preparation and analysis of infected cells (thin section).* To confirm the morphology of VLPs within infected cells, separate cultures infected within the lab were also examined. Samples were prepared for TEM as previously published (Gastrich et al. 2002). Briefly, infected cells were prefixed in a 2% research grade buffered (0.2 M cacodylate, pH = 7.8) glutaraldehyde solution (Thermo Fisher Scientific, Waltham, MA, USA). Then specimens were postfixed in 2% osmium tetroxide (0.2 M cacodylate, pH = 7.8) (Electron Microscopy Sciences), dehydrated, and embedded in EPON™ resin (Hexion Specialty Chemicals, Columbus, OH, USA). Ultrathin sections were collected on copper grids (Electron Microscopy Sciences). *A. anophagefferens* cells were examined for the presence of VLPs using methods previously described (Gastrich et al. 2002).

*Characterization of virus attachment to* A. anophagefferens *cultures.* To determine which particles (if any) associated with *A. anophagefferens* cells during the infection process, we exposed naïve cultures (never having been exposed to a virus) of this pelagophyte to 0.2 μm filtered lysates that had been generated by exposure to the lytic agent. Briefly, 1 mL of lysate (treatment) or 1 mL of killed lysate (control) was added to 25 mL of actively growing culture, and the mixture incubated under standard growth conditions for 30 min to allow for initial attachment. To prevent further infection and in an attempt to immobilize attached particles on the surface of host cells, cultures were fixed with glutaraldehyde (2% v/v final; TEM grade 70% glutaraldehyde from Ladd Research Industries, Williston, VT, USA) buffered in 0.05 M cacodylate 28 psu seawater (cacodylic acid sodium salt from Ladd Research Industries). Preserved samples were immediately placed on

ice and allowed to settle overnight at 4°C (Nudelman et al. 2006). Subsequently, these samples were prepared for SEM. Excess fixative was gently vacuumed off, and samples were washed in water and transferred onto freshly glow discharged and poly-l-lysine coated glass coverslips. Samples, attached to coverslips, were dehydrated in a graded acetone series (25%, 50%, 75%, 90%, 100%, 20 min each step) and critical-point-dried in liquid $CO_2$ with a Ladd Research Industries critical point dryer. Prior to viewing, the coverslips were attached to aluminum specimen stubs with double-sided copper tape and coated with a thin layer of gold in a Structure Probes Inc. (SPI) sputter coater (West Chester, PA, USA). Samples were viewed using a LEO 1525 field emission scanning electron microscope (now under Carl Zeiss MicroImaging Inc., Thornwood, NY, USA) using 3–5 kV accelerating voltage. All images were recorded digitally.

## RESULTS

*Growth curves.* To confirm the use of FSUs as an accurate means to assess *A. anophagefferens* cell abundance and to establish a means to estimate cell number based on FSU readings, culture samples at different times in their growth were enumerated just after fluorescence readings were taken. Plots of cells per mL versus FSUs clustered around a straight line with an $r^2 = 0.970$, indicating a strong correlation between cell number and fluorescence and eliminating concerns over the effects of infection on the use of this biomass measurement. Figure 1 shows the corresponding fluorescence measurements over time of cultures exposed to filtered or unfiltered lysates (Fig. 1A) or controls to which no lysate was added (Fig. 1B). In all experiments, the control tubes, whether they received no viral lysate or killed lysate, were unhindered in their growth. All cultures that received active lysate and were allowed to progress unhindered (i.e., not sacrificed) were eventually completely lysed without recovery. We noted that lysis consistently began between 60 and 72 h postinoculation. Subsequent transfers of these virus lysates (after 0.2 μm filtration) to naïve cell populations have consistently lead to lysis during the past 3 years (more than 20 generations).

*TEM of free virus and infected cells.* To examine the morphology of the infectious agent, a sample of lysate was prepared as described above (negative stain) and examined using TEM. Although the grids were not densely crowded, likely due to allowing the particles to attach randomly to the film via Brownian motion rather than by being spun down with centrifugal force, VLPs could be seen. The particles viewed in this manner were large (slightly <200 nm in diameter), and those that could be clearly seen displayed an icosahedral or nearly icosahedral shape (the latter likely explained as particles that collapsed without being completely flat, i.e., folded over on themselves). Figure 2 shows a representative particle. No tail-like structures were observed attached to any of the VLPs viewed. Examination of *A. anophagefferens* cells infected with lysate as described above (thin section) via TEM revealed large (~140 nm) VLPs of icosahedral shape packed within the walls of the algal cells (Fig. 3). These viruses were consistent with both the free viruses observed by TEM (above) as well as VLPs observed within cells from natural populations (Gastrich et al. 2004).

*SEM of surface attachment.* In the samples analyzed via SEM, large (>100 nm in diameter) icosahedral-shaped VLPs were found only in the treatment sample and not the control (Fig. 4). Very small, potentially phage-like particles were observed in both control and treatment samples, but in neither case could any be seen clearly attached to *A. anophagefferens* cells. Within the treatment group, 66% of the *A. anophagefferens* cells examined had

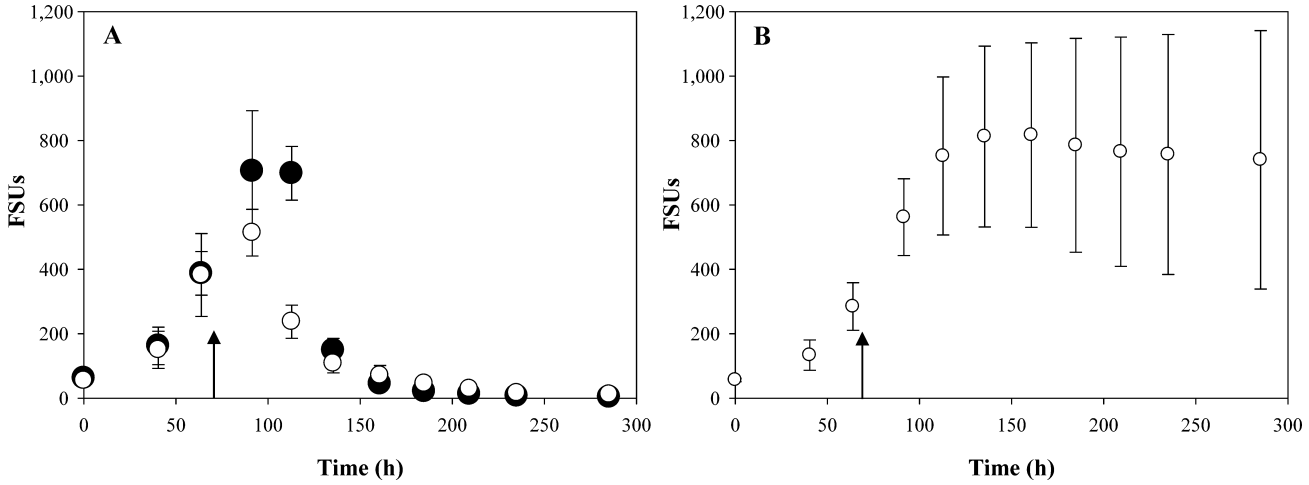


FIG. 1. Growth curves of virus lysate-infected *Aureococcus anophagefferens* cultures. (A) *A. anophagefferens* exposed to unfiltered (open circles) and 0.2 μm filtered (dark circles) water samples containing the putative lytic agent (±SD, $n = 3$ per treatment). (B) Control growth of *A. anophagefferens* cells (±SD, $n = 9$). FSU = fluorescence spectral units. Arrows indicate the addition of 100 μL of virus lysate (A) or 100 μL of sterile medium (B).

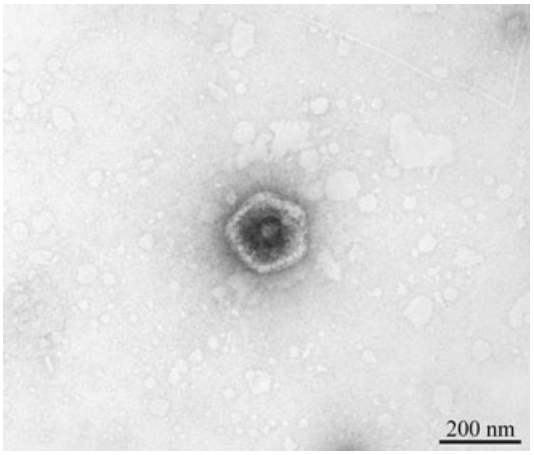


FIG. 2. Negatively stained image of a virus-like particle associated with lysates of *Aureococcus anophagefferens*.

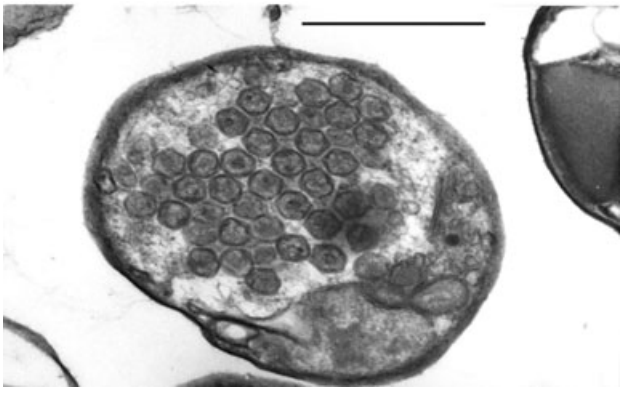

FIG. 3. Thin-section image of *Aureococcus anophagefferens* cell exposed to 0.2 μm filtered lysates. Virus-like particles in cells are similar to those seen in field populations (Gastrich et al. 2004). Scale bar, 1,000 nm.

at least one icosahedral-shaped VLP (~140 nm) attached to their surface after only 30 min. Free VLPs matching the same description were also observed in the treatment group, but again, none were found in the control group.

## DISCUSSION

Interest in the ecological implications of virus infection in aquatic environments has grown significantly in the past few decades as realizations of the potential role of viruses on system geochemistry (Wilhelm and Suttle 1999, Poorvin et al. 2004) as well as diversity and lateral gene transfer (Weinbauer 2004, Suttle 2005) have become more clear. Moreover, the advent of molecular tools has made the correct identification of viruses infecting cells important, as the construction and validation of these tools requires appropriate positive controls

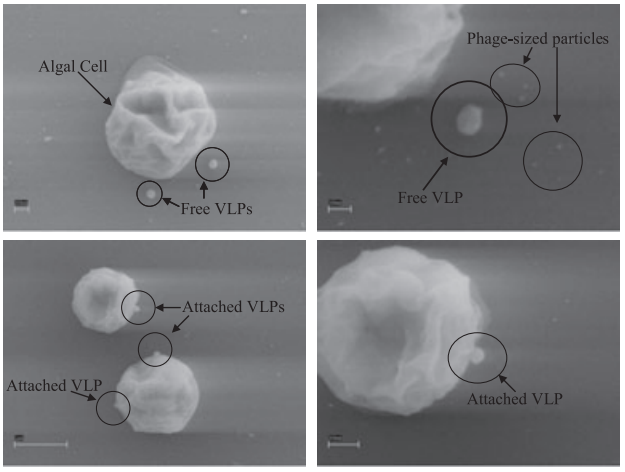


FIG. 4. Scanning electron micrographs of naïve *Aureococcus* cultures exposed to virus-mediated lysates. After 30 min of exposure, the majority of the virus particles appear associated with *A. anophagefferens* cell surfaces (upper left: scale bar, 200 nm; upper right: scale bar, 1,000 nm; lower left: scale bar, 1,000 nm; lower right: scale bar, 200 nm). VLP, virus-like particle.

(i.e., the isolated virus of interest), which must first be accurately identified.

Koch's postulates, the standard that most microbial isolations must meet, were originally developed for confirming disease-causing agents in the medical field (Koch 1884). While they have been well applied in the medical field, they are more difficult to apply to the field of environmental microbiology. Here, to the best of our abilities, we attempted to apply Koch's postulates in confirming the identity and some basic characteristics of a naturally occurring virus infectious to *A. anophagefferens* CCMP 1784. We have in this study been able to show that the virus is present in many cases of cell death, but note that other agents can also cause death in this culture (e.g., bacteria as per Frazier et al. 2007). Furthermore, although the virus can be separated from the host, we have been unable to purify it away from contaminating phage. The virus size-class can, however, subsequently lead to death when new cells are exposed to the isolated agent, and the agent can be repeatedly isolated away from the host and used to reinfect future cell cultures (with appropriate controls). As such, while it is not possible to strictly satisfy these Koch's postulates as originally written, we feel this approach has built sufficiently upon previous studies (Gastrich et al. 2002, 2004) and that it has satisfied the postulates in sensu.

First, we established that a particle susceptible to autoclaving, but not to dilution, caused nonrecoverable cell death of *Aureococcus* cultures, suggesting a living particle and not a toxin. We then used TEM to reveal a potential agent of cell lysis (negative stain described above) and used the morphology of these particles as criteria to confirm the presence of these same particles in later experiments. Because

the smaller phage-like particles were present in both diseased and healthy cultures, they were eliminated from the pool of potentially infectious agents based on Koch's first postulate. The larger VLPs were the only particles we observed to be present in the diseased cultures, and the introduction of VLPs to healthy cultures repeatedly led to infected cells and lysis (collective experiments). Particles of similar size and shape were found only in treatment cultures—free and attached to the algal cell surface in freshly inoculated cultures (SEM described above) and packaged together and often densely within cells at later stages of infection (TEM thin section described above). The reisolation of these disease/lysis causing agents was demonstrated through their appearance in separate experiments, passed on by use of the filtered lysate of the previous experiment. This infection and reisolation has been completed at least 20 times during the past 3 years, with lysates used to infect new naïve cells (data not shown). Finally, EM also allowed us to confirm the viral nature of the pathogen, instead of relying on assumptions based on its filterability. To our knowledge, this is the first report of the use of SEM to demonstrate that a VLP specifically associates itself with *A. anophagefferens*.

Systematic observations of *Aureococcus* cells collected from natural bloom events over the past decade have consistently demonstrated that viruses play a potentially important role in the termination of blooms (Gastrich et al. 2002, 2004). These experiments collectively point to a virus-host system in which the VLP that associates with the host is large (~140 nm) and icosahedrally shaped. It remained somewhat surprising, however, that the isolated particles described in previous publications (Milligan and Cosper 1994, Garry et al. 1998) were so different from the particles observed inside cells. Not only were there variations in morphology (the observation of icosahedral particles inside cells vs. putative isolated particles that were tailed and bacteriophage-like), but there was also a great discrepancy in capsid size (~140 nm size particles within infected cells vs. ~50–55 nm size capsid heads in isolated viruses). The isolation of an axenic culture of *A. anophagefferens* (Berg et al. 2002) should allow for algal viral interactions of this species to be examined without bacteria in the future and thus eliminate the propagation of any incidental bacteriophage.

The isolation of this virus in parallel with the isolation of bacteria capable of lysing *A. anophagefferens* (Frazier et al. 2007) provides a cautionary tale of the difficulties associated with identifying algal pathogens and conducting research with nonaxenic algal cultures. Significant replication as well as the application of companion techniques within our current work allowed us to distinguish between these different agents of mortality. Moreover, these results support the contention that the bacteriophage-like particles identified in previous work may have been microbial hitchhikers—viruses infecting companion or algicidal bacteria that persisted in low abundance in the cultures used in those studies.

The results of this study also provide some clarity in terms of the potential role of viruses in the termination of blooms of *A. anophagefferens*, as they confirm the presence of lytic agents in natural systems that are responsible for the formation of virus particles seen in previous studies (Gastrich et al. 2002, 2004). It remains doubtful, though, that such pathogens will ever be applicable to the control of bloom events. This contention is a function of the Red Queen Theory (Valen 1973) as it applies to virus ecology; host cell populations continue to evolve toward resistant states, even while the viruses continue to evolve toward new infectious states. Our results may help in addressing this as they will allow for the development of better tools (e.g., molecular markers) for monitoring the ontology of brown tides as well as the mortality mechanisms associated with their control and demise. Already the molecular characterization of this virus is underway. Our preliminary PCR results (data not shown), using the AVS1, AVS2, and POL 1 primers described by Chen and Suttle (1995) with the basic protocol described by Short and Suttle (2002), did not indicate that this virus was a member of the Phycodnaviridae family. However, because of its morphology and activity against a eukaryotic alga, we believe that our preliminary results cannot exclude it from such a classification.

This research was supported, in part, by EPA (ECOHAB) Star award #R82-9367-010 to M. D. G., O. R. A., C. J. G., and S. W. W. This is Lamont-Doherty Earth Observatory Contribution Number 7121. We thank C. A. Suttle for screening viruses with the ASV probe set.

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